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Alexis L. Perry
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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|---|---|---|---|
| Applicant: | Tocque et al. | Confirmation No.: | 2833 |
| Serial No.: | 10/070,297 | Art Unit: | 1634 |
| Filed: | March 5, 2002 | Examiner: | Bradley L. Sisson |
| Customer No.: | 21559 | | |
| Title: | METHODS AND COMPOSITIONS FOR DETECTING PATHOLOGICAL EVENTS | | |

Mail Stop Amendment
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DECLARATION OF DR. FABIEN SCHWEIGHOFFER UNDER 37 C.F.R. § 1.132 TRAVERSING GROUNDS OF REJECTION

I, Fabien Schweighoffer, declare:

1. I am a named inventor of the subject matter claimed in United States Patent Application Serial No. 10/070,297 filed on March 5, 2002.

2. I am a Ph.D. in Molecular and Cellular Biology at the Université Pierre et Marie Curie, Paris, France. I am currently Vice President Research at Exonhit Therapeutics SA. I have been working in the field of cell signaling in connection with cancer and neurodegernation for over 16 years.

3. I have read and understood the Office Action, dated November 18, 2005. This Declaration is presented to overcome the rejection of claims 27, 29-33, 44, and 46 under 35 U.S.C. § 112, first paragraph, for lack of written description and enablement.

4. Scientists working under my direction have conducted experiments using the methods described in the specification and known in the art on or before September 16, 1999, to successfully and reliably remotely detect a pathological condition (bovine spongiform encephalopathy; BSE) in a test subject using nucleic acid molecules obtained from blood cells of the test subject.

5. The materials and methods employed are provided by the instant specification or were known to those skilled in the art at the time the application was filed and were as follows:

**Materials**

*Sample material*

To identify specific splicing markers in the early stage of the disease blood samples of 5 experimentally infected cattle and 4 matching control animals were taken. BSE infected cattle in the early stage of the disease were experimentally infected by a single feeding of 1 g of brain homogenate from BSE infected animals in the late stage of the disease.

To identify BSE specific splicing markers in the late stage of the disease, blood samples from three BSE infected animals with severe clinical signs and a positive post-mortem BSE test result and from four healthy controls were obtained from the Veterinary Laboratory Agengy (VLA, Weybridge, UK).

*Blood sampling*

Fifty milliliters (50 ml) of whole blood samples of cattle were anticoagulated with EDTA and immediately mixed vigorously with 100 ml pre-warmed (37°C) RNA/DNA Stabilization Reagent for Blood/Bone Marrow (Roche Diagnostics, Mannheim, Germany). Cells were lysed for 10 min. at room temperature and shock frozen in liquid nitrogen. The blood lysates were stored at -80°C and shipped on dry ice.

*RNA preparation from lysate of whole blood*

One hundred and fifty milliliters (150 ml) of whole blood lysate was thawed by addition of 150 mg dithiothreitol and 100 ml Lysis buffer from MagNA Pure LC RNA Isolation Kit – High Performance (Roche Diagnostics Mannheim, Germany). Automated total RNA preparation was performed with Roche MagNA Pure LC Instrument (Roche Diagnostics, Mannheim, Germany) using a laboratory internal purification protocol. The RNA was recovered in 100 µl elution buffer.

For concentration of the samples, the RNA was precipitated with 2.5 volume 100 % ethanol, 0.1 volume of 3M NaAc (pH 5.2) in the presence of glycogen. The resulting pellet was resuspended in 3 µl water. An aliquot of this sample was run on a 1 % non-denaturating agarose gel to check RNA integrity. As a standard, 90, 45 and 22 ng of HeLa total RNA (Stratagene, La Jolla, California, USA) was used.

*Pooling of RNA samples and mRNA preparation*

To identify the discriminating splicing effects using DATAS™, total RNA preparations from 3 BSE infected cattle in the late stage of the disease and 4 healthy control animals were separately pooled. In a second, independent DATAS™ setup, 5 individual RNA samples from cattle in the early stage of the disease (experimentally infected) and 4 matching, healthy control animals were used. Prior to the DATAS™ experiment, mRNA was prepared from each individual RNA pool using Dynabeads Oligo $(dT)_{25}$ (Dynal) according to manufacturers protocol.

*DATAS™*

DATAS™ was employed as shown in Figure 1. cDNA was synthesized from pooled mRNA samples using SuperScript Preamplification system and first strand cDNA synthesis protocol (Invitrogen, Cergy Pontoise, France) using an anchored biotinylated oligodT primer (Invitrogen, Cergy Pontoise, France).

Two individual libraries were obtained by DATAS™: (1) 1 µg mRNA from blood cells of infected animals (either naturally or experimentally infected cattle) and 500 ng cDNA from

blood cells of healthy controls, (2) 1 μg mRNA from blood cells of healthy controls and 500 ng mRNA from blood cells of infected animals. DATAS™ according to scheme (1) produces splicing variants unique to the diseased samples, while DATAS™ according to scheme (2) produces splicing variants unique to the healthy samples. cDNA and mRNA were hybridized and the resulting mRNA sequences discriminating BSE infected and non-infected animals (i.e., the "RNA loops"; see Fig. 1) were isolated.

Subsequently the RNA loops were cloned using the TOPA-TA cloning kit (Invitrogen, Cergy Pontoise, France) according to the manufacturer's protocol. The vectors were transformed in TOP10 *E. coli* bacteria (Invitrogen, Cergy Pontoise, France), amplified, sequenced and analyzed by nucleic acid database searches.

We identified a total number of 1824 (early stage of the disease) and 1776 (late stage of the disease) redundant cloned alternative spliced mRNA sequences between BSE infected animals and control animals (see "clones in total" in Figure 2). These sequences corresponded to 456 non-redundant sequences (from experimentally infected animals) and 275 non-redundant sequences (from naturally infected animals; see "non-redundant sequences" in Figure 2).

Out of these mRNA sequences all 336 mRNA sequences cloned one time (singletons) were used for microarray analysis (109 singletons identified by DATAS™ on naturally infected and 227 identified in the study based on experimentally infected cattle). Additionally 482 sequences were selected from the group of sequences cloned more than one time (redundant sequences). In total 818 unique sequences were identified and used for microarray analysis.

*Microarray analysis*

Clones of 818 unique sequences transformed in TOP10 *E. coli* bacteria were grown overnight. A primary PCR was carried out for pre-amplification of vectors. The PCR product resulting from the primary PCR was used in a subsequent PCR to enhance specificity of the PCR product. The whole volume after the second PCR was concentrated by precipitation. The PCR product was checked by agarose gel electrophoresis for product size.

PCR products of selected clones and controls were spotted on a glass slide. As reference, a pool of bovine RNA from lung, kidney and liver (1/3 each) was used.

Synthesis of cRNA from mRNA was performed in four steps. (1) Synthesis of double stranded DNA, (2) clean-up with Phase Lock Gels (Eppendorf), (3) in vitro transcription of cRNA using a T7 promotor side and Megascript T7 amplification kit (Ambion) and (4) clean-up of cRNA using RNeasy Mini Kit (Qiagen). cRNA quantification was performed with Agilent 2100 Bioanalyser (Agilent Technologies).

Five to six μg of cRNA were Cy3 and Cy5 labeled (Amersham) and the labeled product was purified with Microcon Y-30 (Millipore). The labeling reaction was checked by optical density measurement at 260 nm (cRNA), 550 nm (Cy3), and 660 nm (Cy5). Cy3-labeled: 3 samples of experimentally infected and 3 non-infected animals, 6 samples of naturally infected and 5 non-infected animals.

Two μg of labeled cRNA of individual infected and non-infected control animals were hybridized with glass slides of selected clones (see figure 3 for an example of such an hybridization).

Statistical array data evaluation was performed using SAM software (Significance Analysis of Microarrays, http://www-stat.stanford.edu/~tibs/SAM/index.html)to identify differentially expressed genes. PAM software (Prediction Analysis of Microarrays, http://www-stat.stanford.edu/~tibs/PAM/index.html) was used to predict the status (infected or non-infected) of a cow based on the splicing marker profile.

6. The methods described in paragraph 5 produced the following results:

Using DATAS™, we identified a total number of 1824 (early stage of the disease) and 1776 (late stage of the disease) redundant cloned alternative spliced mRNA sequences between BSE infected animals and control animals (see "clones in total" in Figure 2). After reduction of redundancyt, a total 818 unique sequences were identified and used as the nucleic acid library for microarray analysis.

The 818 markers identified by DATAS™, which represent a library of differentially spliced nucleic acid molecules between the blood cells of animals infected with BSE and control animals, were applied to a glass slide. Labeled cRNA from individual infected and non-infected animals were then separately hybridized to the nucleic acid library on the glass

slides. Hybridization in each case was then analyzed by significance analysis of microarrays (SAM) and prediction analysis of microarrays (PAM). These statistical analyses revealed, in particular, a set of 5 splicing markers (LT1-LT5) that discriminated between BSE infected and non-infected cattle, regardless of whether the infection was at an early or late stage (see figure 4). Even with blood samples of animals in the early stage of the disease, the p-values of the t-test show significance for these markers. According to SAM and PAM analysis, these five markers are sufficient to discriminate between BSE infected animals in the early or late stage of the disease and healthy animals. Since alternative splicing markers will only occur when the splicing apparatus is significantly changed, e.g., by the occurrence of a pathological condition, these splicing markers represent a more accurate method of detecting pathological conditions in patients than the use of mRNA expression markers that might be controlled by different short-term changes (e.g. circadian cycles and nutrition).

7. The data clearly demonstrate that splicing markers (i.e., the library) identified from whole blood samples of animals having BSE (i.e., a pathological condition associated with a deregulation of a cell signaling pathway) can be used to determine whether a test subject has that pathological condition. By simply preparing nucleic acid molecules from blood cells of the test animal (i.e., the test sample) and applying them to the library affixed to a glass slide, we were able to detect the presence of the pathological condition in infected test animals based on the hybridization of their nucleic acids with the nucleic acids of the library and to identify non-infected healthy animals based on the absence of hybridization of their nucleic acids with the nucleic acids of the library. Thus, the methods of the invention can be used to successfully and reliably confirm the detection of BSE, , and other pathological conditions associated with a deregulation of a cell signaling pathway, using nucleic acids obtained from blood cells.

8. The data clearly demonstrate the remote *in vitro* detection, using only blood cells from a test subject, of the presence of a given, predefined pathological condition, BSE, according to the methods of present claims 27, 39-33, 44, and 47-49. The data demonstrate the successful preparation of a nucleic acid library characteristic of the pathological condition,

using art-known techniques, and the reliable detection of that pathological condition by detecting hybridization between the nucleic acid molecules of the library and nucleic acid molecules obtained from a sample of blood cells from the test subject (i.e., the hybridization profile). This methodology can be used to detect any pathological condition characterized by alternative splicing events in blood cells as a result of the pathological condition. Moreover, the results disclosed above for detecting the presence of BSE in infected animals are predictive of success for detecting pathological conditions characterized by alternative splicing events in humans.

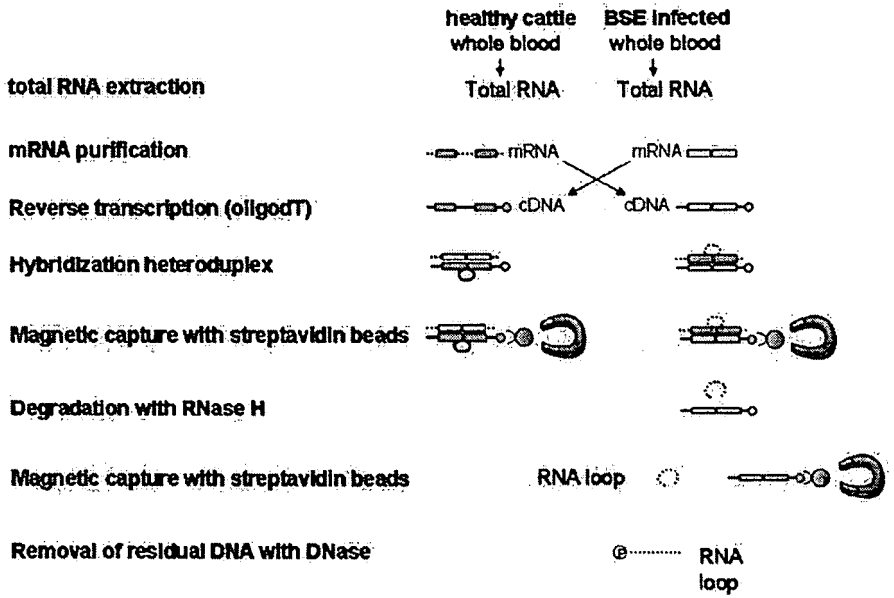
9. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Respectfully submitted,

Date: May 11th, 2006

Dr. Fabien Schweighoffer

Figure 1



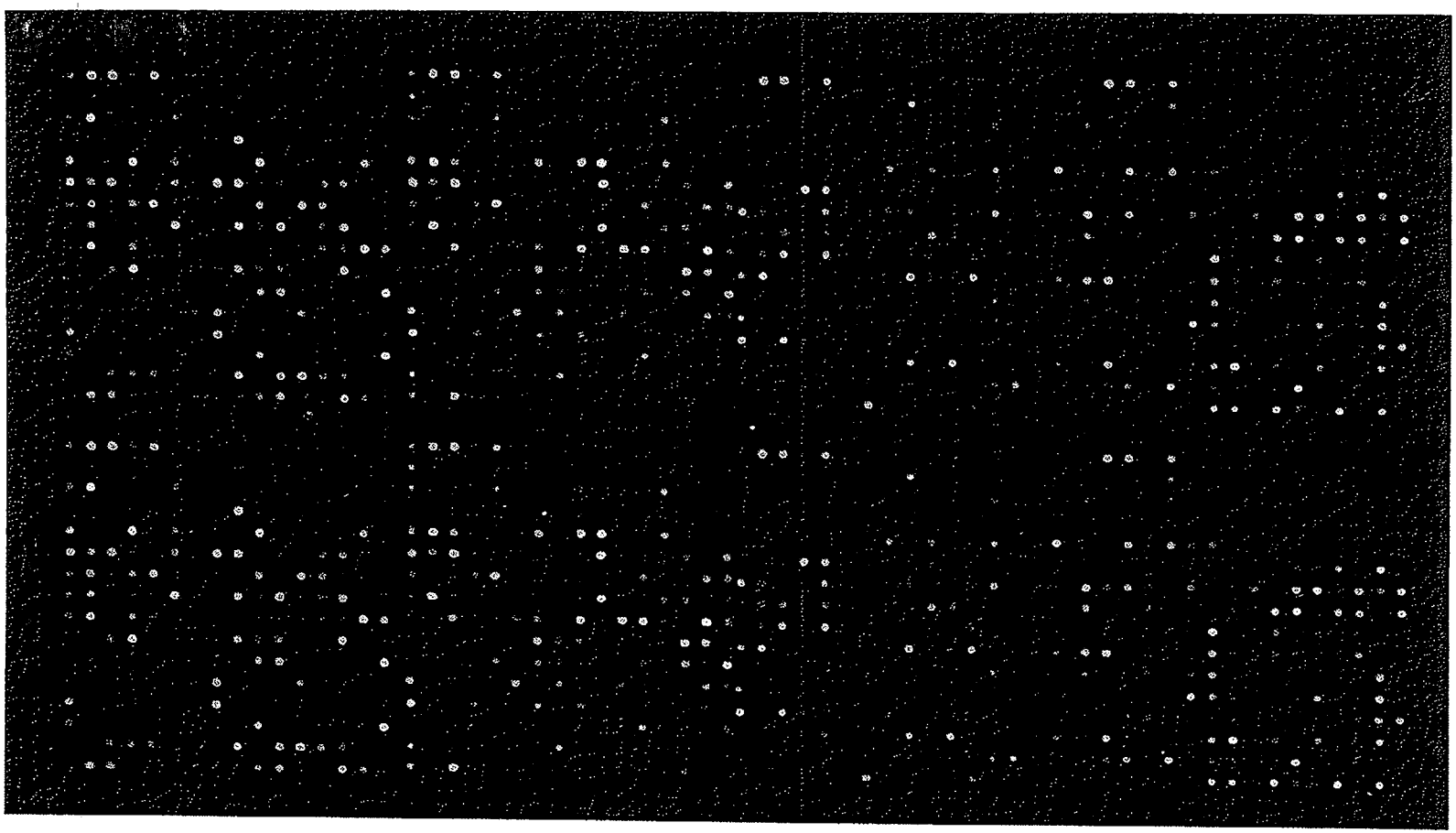

Experimental workflow of DATAS™. mRNA prepared from whole blood samples of BSE infected and healthy cattle are reverse transcribed based on oligodT primers. The resulting cDNA is biotinylated. By heteroduplex hybridization of mRNA and cDNA, nucleotide sequences differing between the two conditions form unhybridized loops. The heteroduplexes are captured with streptavidin beads and digested with RNase H, which specifically digests RNA molecules in double strand with cDNA. The remaining RNA loop (i.e., the alternatively spliced region) is purified and the remaining DNA is digested prior to cloning of the RNA.

Figure 2

| a) | nat.inf. | exp.inf. |
|---|---|---|
| Clones in total | 1776 | 1824 |
| Sequences screened out | 445 | 151 |
| Sequences in clusters | 1222 | 1446 |
| Number of clusters | 166 | 229 |
| Singletons | 109 | 227 |
| non-redundant sequences (number of clusters + singletons) | 275 | 456 |

| b) | nat.inf. | exp.inf. |
|---|---|---|
| non-redundant sequences | 275 | 456 |
| mitochondrial genome sequ. | 6 | 13 |
| genomic sequences | 77 | 12 |
| sequ. not found in databases | 64 | 102 |
| EST | 43 | 130 |
| Others | 85 | 199 |

Figure 3



Example of a microarray regrouping the 818 sequences co-hybridized with target derived from an infected cow labeled in Cy3 and a reference target labeled in Cy5

Figure 4

| Marker | infected score | non-infected score |
|---|---|---|
| LT1 | -0.2419 | 0.2722 |
| LT2 | -0.1847 | 0.2078 |
| LT3 | -0.1823 | 0.2051 |
| LT4 | -0.1541 | 0.1734 |
| LT5 | -0.1459 | 0.1641 |

Statisticalanalysis of array experiments of nucleotide sequence markers of both DATAS™ experiments (early and late stage of the disease). PAM scores for infected and non-infected groups are shown.